Use of Tetraphenylethylene in Quantitative GLC of Hyoscyamine and Scopolamine

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Abstract □ Hyoscyamine and scopolamine as free bases were simultaneously quantitated by GLC, using tetraphenylethylene as an internal standard. A linear relationship was found between the ratios of the integrated peak areas of alkaloid to the internal standard and the actual weight ratios of alkaloid to the internal standard for both hyoscyamine and scopolamine. Pure compounds and extracts from *Hyoscyamus niger* Linne powder were analyzed. The combined precision of the extraction procedure of plant materials with the GLC procedure was determined.

Keyphrases □ Tetraphenylethylene, internal standard—quantitative GLC of hyoscyamine, scopolamine □ GLC, hyoscyamine, scopolamine, quantitative—tetraphenylethylene, internal standard □ Hyoscyamine—simultaneous GLC quantification with scopolamine using tetraphenylethylene as internal standard □ Scopolamine—simultaneous GLC quantification with hyoscyamine using tetraphenylethylene as internal standard

Brochmann-Hanssen and Svendsen (1) successfully separated a large number of alkaloid mixtures from various plant sources by GLC. Solomon *et al.* (2) reported quantitative determination of atropine and scopolamine by GLC. No information was available pertaining to the use of a proper compound as an internal standard for the quantitation of the two major tropane alkaloids. Furthermore, no estimate of the combined precision of the alkaloid extraction procedure involving plant materials with the GLC procedure has been reported. Recently, Zimmerer and Grady (3) described an assay procedure of hyoscyamine, atropine, scopolamine, and phenobarbital in unit doses of tablets and elixirs, using homatropine as an internal standard.

The importance of using an internal standard to obtain reproducible quantitative results in any assay procedures by GLC has been emphasized by various investigators (4–9). This paper reports the quantitative determination of hyoscyamine and scopolamine simultaneously as pure compounds and from plant extracts, using tetraphenylethylene (TPE) as an internal standard by employing both isothermal and programmed temperature GLC.

EXPERIMENTAL

Equipment—A linear programmed temperature gas chromatograph (Perkin-Elmer model 881), equipped with a hydrogen flame-ionization detector and a 1-mv. recorder (Sargent model SR, S-72180-20) with a chart speed of 1 in./min. and 1-sec. full-scale response, was used. A pH meter (Beckman zeromatic), centrifuge, and flash evaporator were used for the extraction of plant powders.

Materials—The carrier gas was helium. Hydrogen and air were used in the flame-ionization detector. Dual borosilicate glass columns, 1.83-m. (6-ft.) × 0.19-cm. (0.075-in.) inside diameter, were packed with 2.5% of methyl silicone gum rubber[1] on diatomite aggregate,[2] DMCS 80/100 mesh. The prepared packing material (Perkin-Elmer Co.) was introduced into the columns under reduced pressure with uniform vibration. A minimum of Pyrex glass wool was used to hold the packing material in place in the column. The columns were conditioned and maintained by the injection of Silyl-8[3] into the chromatograph once every 2 weeks before any sample analysis was made. Seeds of *Hyoscyamus niger* Linne (Solanaceae), annual variety, were used.[4] Macroscopic and microscopic examination of aerial organs of flowering plants in this laboratory confirmed identity as hyoscyamus NF XI (henbane). Plants which were field grown, oven-dried at 50°, and ground to 40 mesh served as the standard plant powder. Hyoscyamine[5] and scopolamine[6] free bases, as well as their hydrobromide salts,[7] were used. TPE was also used.[6] All other chemicals used were analytical reagent grade.

Operating Conditions—The sample was chromatographed isothermally at 200° for 6 min., followed by programmed temperatures from 200 to 290° at the rate of 6°/min. The injector temperature was maintained at 300°. The helium flow rate was 100 ml./min., with an inlet pressure of 24 psig. Air and hydrogen inlet pressures were 48 and 24 psig., respectively. Attenuations of ×100, ×50, ×20, and ×10 were used.

Standard Curves—Nine separate chloroform solutions were prepared by weighing out known amounts of hyoscyamine, scopolamine, and the internal standard, TPE, into 10-ml. volumetric flasks. The solutions were chromatographed, and the integrated peak areas of hyoscyamine, scopolamine, and TPE were obtained. A standard curve was then established by plotting the ratio of peak areas of hyoscyamine to TPE *versus* the weight ratio of the two compounds. A similar curve was obtained for scopolamine. On alternate days during this study, fresh solutions of the two alkaloids and the internal standard were used to determine the reproducibility of the standard curves.

Quantitation of the Alkaloids of Hyoscyamus Powder—*Extraction Procedure*—Hyoscyamine and scopolamine were extracted from standard hyoscyamus powder as total alkaloids by the procedure previously described (10).

Preparation of Sample Solution—A stock solution was prepared by dissolving a known amount of TPE in chloroform. A definite volume of this standard TPE solution was added to an aliquot of the plant extract by means of a lambda pipet and thoroughly mixed. The mixed solution was then chromatographed. The usual sample size injected was approximately 2 μl. Pure hyoscyamine and scopolamine as free bases were used to check the reproducibility of the procedure each day that plant extracts were analyzed.

Calculations—The amounts of hyoscyamine and scopolamine in hyoscyamus powder were determined by computing the alkaloid to TPE peak area ratio from the chromatogram, obtaining the cor-

Table I—Gas Chromatographic Data

Parameter	Hyoscyamine	Scopolamine	Tetraphenylethylene
Retention[a]	0.87	(1.00)	1.13
Asymmetry, A_s (11)	1.10	1.16	1.00
Theoretical plates (11)	17,000	19,000	42,000
Response[b]	1.10	(1.00)	2.47

[a] Actual retention time = × 15.1 min.; see Fig. 1. [b] Equimolar amounts, ratio of integrated peak areas.

[1] SE 30/S.
[2] Chromosorb G, acid-washed.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Obtained from Dr. Lynn Brady, University of Washington, Seattle, Wash.
[5] New York Quinine and Chemical Works, Inc.
[6] Aldrich Chemical Co. Inc.
[7] S. B. Penick & Co.

Table II—Simultaneous Determination of Hyoscyamine and Scopolamine in Standard Hyoscyamus Powder

Weight of Plant Powder, g.	Alkaloids Found, mcg.		Alkaloid Concentration, mg./100 g. dry wt.	
	Hyoscyamine	Scopolamine	Hyoscyamine	Scopolamine
4.35	244	544	5.6	12.5
3.54	202	439	5.7	12.4
4.46	236	540	5.3	12.1
4.55	264	573	5.8	12.6
Mean			5.6	12.4
95% Confidence limits (12) of mean			5.3 and 5.9	12.1 and 12.7

Table III—Recovery Data

Weight of Plant Powder, g.	Theoretical Amount,[a] mg.		Amount Recovered, mg.		Percent Recovery	
	Hyoscyamine	Scopolamine	Hyoscyamine	Scopolamine	Hyoscyamine	Scopolamine
4.60	3.27	5.66	3.18	5.39	96.9	94.6
4.36	3.25	5.63	3.30	5.50	101.7	97.5
4.56	3.27	5.65	3.05	5.67	92.9	100.4
4.77	3.28	5.68	3.25	5.86	99.0	103.5
Mean					97.6	99.0
95% Confidence limits (12) of mean					91.7 and 103.5	93.0 and 105.1

[a] Consists of 3.01 mg. hyoscyamine and 5.09 mg. scopolamine which was added volumetrically to each plant sample, alkaloid content of which was calculated from data in Table II.

responding weight ratio of alkaloid to TPE from the standard curve, and multiplying by the weight of TPE in the sample. The value so obtained was then converted to alkaloid concentration in mg./100 g. dry weight of powdered plant specimen.

Recovery Studies—A solution of hyoscyamine hydrobromide and scopolamine hydrobromide as a mixture was prepared by dissolving known amounts of the two alkaloid salts in 10 ml. distilled water. An aliquot of this solution was added to a weighed hyoscyamus powder sample and extracted and analyzed by this method to estimate the recovery of the added pure alkaloids.

RESULTS AND DISCUSSION

For accurate quantitative analysis, the internal standardization technique was used. The virtues of using an internal standard have been well established (3–9).

Figure 1 is a typical gas chromatogram of a mixture of pure hyoscyamine and scopolamine, with TPE added as an internal standard. Extracts of *Hyoscyamus niger* L. with TPE added showed similar chromatographic characteristics. The three compounds were well separated from one another with no overlapping, and TPE was found in the proximity of hyoscyamine and scopolamine. Therefore, in these studies, TPE can be used as an internal standard for each pure alkaloid individually, as a mixture, or in plant extracts. Moreover, TPE is readily available commercially, does not exist in plant extracts, remains stable in chloroform solution for relatively long periods of time, and does not decompose within this operating temperature range of GLC. Various other compounds such as acetanilid, pilocarpine, and pilocarpine hydrochloride were investigated in this study for their ability to serve as an internal standard; however, TPE proved to be the most satisfactory.

Brochmann-Hanssen and Svendsen (1) separated hyoscyamine and scopolamine from hyoscyamus leaf extract by GLC at 200° isothermally. Solomon *et al.* (2) reported a GLC procedure involving temperature programming only from 150 to 275° at the rate of 6°/min. for the separation of these two alkaloids from plant extracts. During initial experimentation, both procedures were compared using certain root extracts of *H. niger* as the sample. Data obtained showed the presence of a few compounds eluted between the solvent

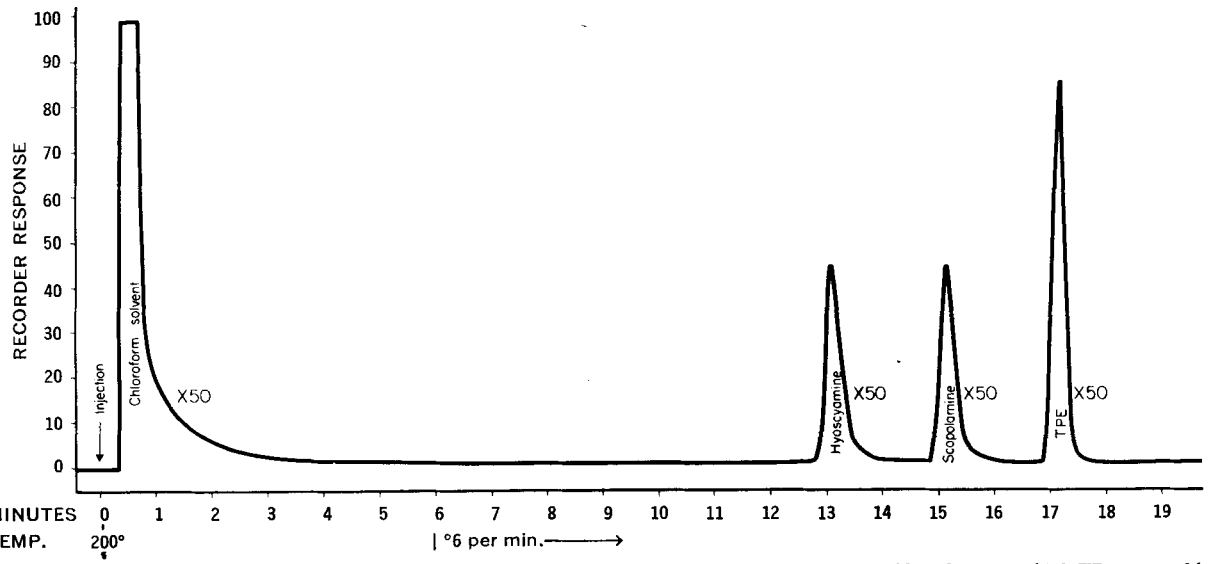

Figure 1—*Typical gas chromatogram of a mixture of pure hyoscyamine and scopolamine bases in chloroform to which TPE was added as the internal standard. Extracts of* Hyoscyamus niger *L. with TPE added showed similar chromatographic characteristics.*

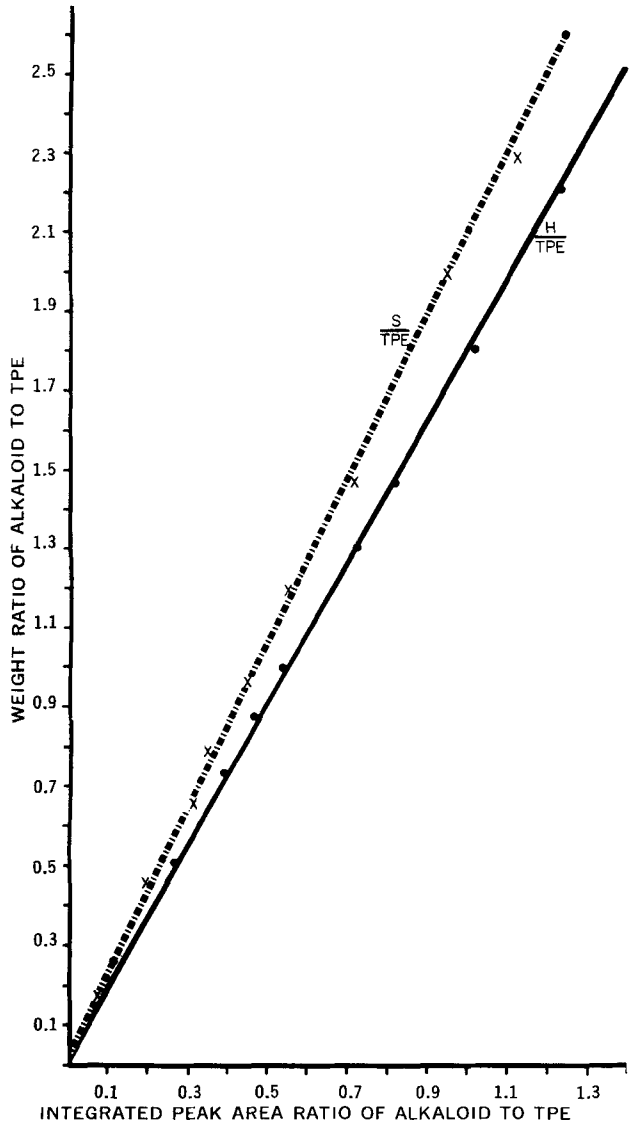

Figure 2—*Standard curves for hyoscyamine and scopolamine with TPE as the internal standard.*

peak and the hyoscyamine peak, resulting in chromatograms that did not afford the desired resolution. A technique was desired where the two alkaloids in question would have greater retention times and also greater differences in retention time so as to minimize or eliminate any possible interfering effect of the solvent and/or any other substances on the quantitation of hyoscyamine and scopolamine. A combination of both isothermal and programmed temperature GLC yielded well-separated symmetrical peaks (Table I). The number of theoretical plates suggested high column efficiency. The resolution factors (8) between hyoscyamine and scopolamine and between scopolamine and TPE were 2.2 and 2.4, respectively, indicating complete resolution.

A linear relationship was established between the weight ratios of the alkaloid to the internal standard and the integrated area ratios of the two. Figure 2 shows the standard curves for hyoscyamine and scopolamine with TPE as the internal standard. Maximum precision and accuracy were obtained when the integrated peak area ratio of alkaloid to TPE was near unity. Each value shown in Fig. 2 is the average of three independent determinations. The standard curves thus obtained were checked and redetermined with fresh standard solutions. The use of an internal standard greatly simplified the analytical procedure and calculations.

Samples of hyoscyamus powder were analyzed by this method, and the results are shown in Table II. The combined precision of the liquid–liquid extraction technique with the GLC procedure was examined by adding known amounts of the two alkaloid salts to hyoscyamus powder. The results obtained from the recovery studies are presented in Table III. In all cases, quantitative recoveries of the added alkaloids were obtained.

Brochmann-Hanssen and Svendsen (1) pointed out that the amount of glass wool placed on top of the column packing was closely associated with the degree of decomposition or dehydration of the alkaloids. Special care was taken in using only the minimum amount of glass wool practicable during column packing. As shown in Fig. 1, no additional peaks apparently due to decomposition were present when pure alkaloids or henbane extracts to which TPE was added were chromatographed. The treatment of columns by injection of Silyl-8, which is a mixture of three trimethylsilyl donors, also aids in enhancing the column efficiency and preventing breakdown of sensitive compounds.

It should be emphasized that under the conditions employed, GLC did not differentiate between hyoscyamine and atropine. Zimmerer and Grady (3) arrived at the same conclusion and thus used the term "hyoscyamine-atropine" in their discussion.

The results obtained in this study clearly demonstrate that TPE is an excellent internal standard in the quantitative GLC determination of hyoscyamine and scopolamine, irrespective whether present as authentic free bases, mixtures, or in plant extracts.

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